ANALYTICAL METHOD DEVELOPMENT OF EMBELIN BENZYOL SUBSTITUTED ESTER DERIVATIVES BY USING REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP – HPLC)

A

SYNOPSIS

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Introduction

Natural products are the prime potentials for the drugs, which have been providing novel, clinically and pharmacologically active drugs. The main source of the drug is the plant, for the plant crude drug is extracted using a suitable solvent, isolated by column fractionalisation, purified and characterized for the medical usages.

Embelin is one of the drug extracted form Embelia ribes. The name of the Plant is maintained under the Botany, taxonomy classification is as follows

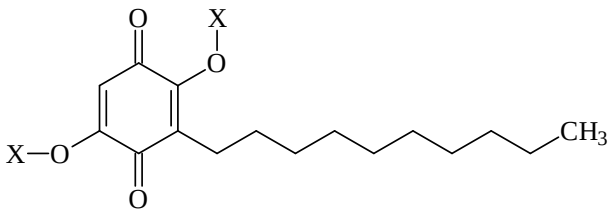
Roder	Ericales
Family	Myrsinaceae
Genus	Embelia
Species	E. ribes

The plants Embelia ribes parts like fruits, leaves and root & stem bark are the main potential of the drug Embelin. The drug is separated, isolated, purified and characterized for its medical property like abdominal disorders, fungal infections, mouth ulcers, constipation and other uses. The Embelin molecule structure has many pharmacological usages. Where as the basis molecular structure of embelin is 1,4-benzoquinone, hence this base molecular structure is kept as it is and the synthesized the substituted benzoyl derivatives. Embelin substituted benzoyl derivative as per **Upadhyay et al (2008)** has more potential or efficacy like parent drug (embelin). Hence the derivatives of Embelin also have to be pay importance to study for its property.

In this present study the derivatives will be studied extensively by using various analytical techniques, Like Validated method for Oseltamivir in Pharmaceutical formulation as per **Nagarajan et al (2009),** Quantitative and Chromatographic fingerprint analysis of Embelia ribes (in churna formulations) by HPLC method as per **Sudani et al (2011),** RP-HPLC Assay Method for the Determination of Memantine HCl Drug Substance with Uv-Vis Detector as per **Bhavil et al (2005)** HPLC, UV-Vis, FT-IR and DSC. The maximum yield of embelin

from its seed at a particular harvesting time (by HPLC method) as per **Pandey et al (2011)** analytical estimation and analytical method Development and validation by RP-HPLC of Embelin along with, Rottlerin and Ellagic acid in Vidangadi churnaas per **Rakesh et al (2012)**. The Embelin benzoyl substituted new derivatives will be synthesized and analytical method will be developed by RP-HPLC and the method will be validated as per the current guideline requirements

A general molecular structure of the substituted benzoyl chloride derivatives will be represented by the following structural formula.



Where the X is fluoro, chloro, bromo, iodo, nitro, methoxy derivative of substituted benzoyl chlorides (ortho, meta and para substituted).

Review of Literature

The literature review of the Embelin is extracted from empelia ribes at a set of specified conditions, which is determined by UV-Vis technique as per **Ganesan et al (2010).** The Embelin will be converted in to its Ester benzoyl substituted derivatives and found that has lowering lips properties. The Embelin is one of the natural drugs used for many medical utility; As per **Radhakrishnan et al (2011)** the natural benzoquinon extract derived from embelia ribes has Antibacterial functions, As per **Srinivas, (2010)** the embelin derivatives has anticancer properties, As per **Jindal et al (2012)** the Antioxidant Activity like embelin has the Ardisia crispa molecule, as per **Poojari et al (2010)** Embelin has Chemopreventive and Hepatoprotective Effects in the presence of on N-Nitrosodiethylamine and Carbon Tetrachloride Induced Preneoplasia and Toxicity in Rat Liver, as per **Radhakrishnan et al (2011)** embelin molecule has cosmetic proprieties in its usage, as per **Prakash et al (2011)** the In Vitro and In Vivo Anticancer Activity of Bacoside A from Plant of Bacopa Monnieiri (Linn), as per **Dai et al (2011)** the Embelin enhances therapeutic efficacy of ionizing radiation in prostate cancer, hence embelin is being used as cancer inhibitor, as per **Maulik et al (2009)** the embelin molecule contains Embelia ribes has the properties of antifungal activity and many others medical usages since long time.

Many of the metallic co-ordinate complexes of embelin are synthesized and characterized. As per **Rani et al (2010)** embelin zinc (II) complex is synthesized called Ichlorobis (Emnbelinate), as per **Rani et al (2010)** Embelin cobalt (II) complex is synthesized and characterized. Hence many other metallic complexes of embelin are being used as specific medical usages. The embelin metallic co-ordination complex also has thermal stability and the co-ordination covalent bond formations are also proven in may studies including the kinetic properties of embelin metal derivatives or meta complexes, as per **Singh et al (2012)** Kinetics and Thermal Decomposition of Sm (III) complex with Embelin (2,5-Dihydroxy-3-Undecyl-p-Benzoquinone) had been studied and found that the thermal decomposition around 245 °C by Differential Scanning Calorimeter (DSC).

In the medical treatment, some of the treatments will be planned for short time duration and few other treatments are being carried out as long time treatment, it is depends up on the drug administrations and the drug kinetic properties. As per

Buchwald et al, (1980) long-term treatments are carried out controlled manner. As per **Drent et al. (1993)** the embelin derivatives has inhibition properties by acting on of lipases molecule at cell levels. In some other usage like lowering the plasma triglycerides as per **Liu et al (1994),** which was tested by alteration of Lipid Profiles in Plasma of Transgenic Mice to Human Lipoprotein Lipase which, increased the lipoprotein density.

The current trend around the world, in human beings is opposing the obesity or over weight, in other words avoiding the unwanted or extra body weight. All the humans are interested to be fit about physically; at the same time hesitate to oblige the diet behavior. In general adoption of a diet containing rich fat and this is being continued in the modern life style for all expect the hard working persons. Such enormous administration of fat containing foods will lead to accumulations of fat continuously in the various parts of body.

All the fat are not being used daily or burned daily, hence the fat is being accumulated; in the various parts and various levels in the body. Since the populations of mankind is being in raising order, the obesity is also being raised as inter related of populations. The obesity and gaining more weights would be genetic properties in the enzyme level as per **Wong et al (2002)** hence many of the genetic disorder provide the body nature like their parents including body weight at their later stages. The human lipoprotein lipase or hepatic lipase have decreased levels of plasma triglycerides and an increased level of high density lipoprotein as per **Krapp et al. (1996)** and **Wong et al (1991),** the outcome of hepatic lipase also alone**.** Lipoprotein lipase has physical dissimilarities due to the irregular metabolism, hence it is believed that the regular. As per **Jaye et al (1999)** metabolism is being processed by the function of lipase. Availability or presence of High-density lipoprotein / fat in the body is inversely related to the cardiovascular disease. Controlling or maintaining of cholesterol level in the body is being balanced by the genetic properties. The metabolism of the high density lipoprotein or fat is being controlled or depends up on the Lipoprotein lipase (LPL) & hepatic lipase (HL), the LPL & HL are belonging from Triacyl Glycerol Lipase (TGL) family. Hence the TGL and related lipase are responsible to the body metabolism, if these are not functioning regular or properly the body metabolism will not be perfect and hence the fats / cholesterol would not be properly re-cycles or utilized, this will led to deposition

of extra cholesterol in the body system. As per **Hixenbaugh et al (1989)** the benzo quinine based (embelin) molecule controls or being influenced may medical activities like overweight, obesity, hyperlipidemia, hypercholesterolemia, hypertriglyceridemia, pancreatitis, diabetes, atherosclerosis, other cardiovascular diseases. The Lipases has the controlling properties of lipid lowering activities, but the maximum lipase are being produced by yeast are available commercially, hence the purities of the lipase is very less; which contains many of the isomers, which is not performing the lipid lowering activities, hence purification of lipase is important as per **Yadav et al (1998)**

The obesity will leads to many associated effects like, extra weight, diabetes, heart (cardiovascular) problems, thyroid and hypertension problems. Hence controlling of the obesity is also being importance task in the present circumstances. Controlling of fat accumulation has to be controlled, in terms of burning the extra energy / fat, through some inhibiting agent or aid participation. The aid / inhibiting agent has to be act at the cell levels. There are two important lipase are used for the accumulation, catabolism and reforming of **Chappell et al (1994), Winkler et al (1979)** lipoproteins along with phospholipids. Hepatic lipase and lipoproteins are very active, multifunctional proteins on the surface of the peripheral tissue cells including on liver cells.

These two hepatic lipase and lipoproteins are called enzymes, which participate in reverse fat (cholesterol) transmission[18] processes. These **Gupta et al (1989)** enzymes will transport the fat from **Faustinella et al (1992)** peripheral tissue to liver; where the liver will do the extraction processes are recycling the cholesterol / fat. There the polymeric or protein lipase will be act to **Langer et al (1984), Langer et al (1983), Langer, (1990)** release the drug in a controlled manner.

The redirected or reversed fats / cholesterol are processed in liver of rat and human, the molecular mechanism is attained as per **Lowe (1997).** The two types of novel Human Pancreatic Lipase Related Proteins, hPLRPl and hPLRP2 Differences in colipase dependence and in lipase activity as per **Giller et al (1992)**. During the metabolism depend on the Glycogen group of lipase and hyaluronate as per **Winkler et al (1990)**. The pancreatic lipase and the other group of the same family activities are elaborated by **Verger (1984);** hence evidenced that the pancreatic acid (liver juice) play a vital role in the processes of eliminating, extracting or recycling of

cholesterol. Where the insulin will be put in to the action by intestinal absorption of very long chain molecules like fatty acid of **Goldberg et al (1988), Goldberg et al (1982)** triglyceride and hence the extra fat will be reduced or controlled by lipid lowering agents. As defined **Giller, T. et a1. (1992)**, Lipolytic enzymes are water insoluble in nature, hence the enzymes will interact on the water-lipid junctions, this nitration will all the area of pancreatic lipases and also on the homologous hepatic and **Shimada et al (1993), Ranganathan et al (1995)** lipoprotein lipases inter-phase junctions.

Embelin is one of the natural lipid lowering agents by the base molecule benzoquinon and its hydroxyl group along with its 11 carbon chain. The modification of the molecules by substitution on its hydroxyl group will be derived in a general name as substituted benzene derivatives of embelin molecule.

The following molecules are taken up for the lipid lowering proprieties study.

2,5-Di-O-(4-chlorophenylcarbonyl)-3-undecyl-1,4-benzoquinone (RLS 33)

2,5-Di-O-(2-bromophenylcarbonyl)-3-undecyl-1,4-benzoquinone (RLS 34)

2,5-Di-O-(4-bromophenylcarbonyl)-3-undecyl-1,4-benzoquinone (RLS 36)

2,5-Di-O-(2-iodophenylcarbonyl)-3-undecyl-1,4-benzoquinone (RLS 37)

2,5-Di-O-(3-fluorophenylcarbonyl)-3-undecyl-1,4-benzoquinone (RLS 38)

2,5-Di-O-(3-nitrophenylcarbonyl)-3-undecyl-1,4-benzoquinone (RLS 40)

2,5-Di-O-(2-fluorophenylcarbonyl)-3-undecyl-1,4-benzoquinone (RLS 41)

2,5-Di-O-(4-Nitrophenylcarbonyl)-3-undecyl-1,4-benzoquinone (RLS 42)

2,5-Di-O-(3-methoxyphenylcarbonyl)-3-undecyl-1,4-benzoquinone (RLS 44)

2,5-Di-O-(4-methoxyphenylcarbonyl)-3-undecyl-1,4-benzoquinone (RLS 45)

(Where the RLS is notation is short note for identification or reference in short form)

All these molecules will be subjected to analysis by HPLC for determining the Quantification method.

Objectives

Study to develop a Reversed Phase High Performance Liquid Chromatography for separation and determination of substituted benzoyl ester derivatives of embelin molecule.

To separate and quantify ten different isomers substituted benzoyl ester derivatives of embelin molecule by a single RP-HPLC run.

The new RP-HPLC method for substituted benzoyl ester derivatives of embelin molecule will be valued in terms of simple, accurate, specific and cost effective for routine analysis.

Work Plane

The samples were subjected to the extensive analytical techniques, for its molecule confirmations and these molecules will be used as standard in this study.

HPLC Method Development Requirements:

The method shell be developed for the separation of the Embelin derivatives; this processes will be considered the following property of the molecules.

1 Charge (Positive/negative of the molecule to be separated)
2 Hydrophobicity (of the solute and the solvent used in the analysis)
3 Affinity (of the solute and the solvent used in the analysis)
4 “lock and key” (sites of the column used in the analysis)
5 Solubility & stability (solute solubility and the stability of the solution)
6 pH, ionic strength, (organic solvents and buffers used in the analysis)
7 Molecular weight (derivative molecule)

After studying for the above mentioned properties of the solute and solvent, the separation of the derivatives will be achieved through a suitable solid support column.

The separation is subjected to the following study the analytical evolutions:-

l Resolution (degree of separation between main peaks and other impurities)
2 Band spreading (selectivity)
3 Recovery (mass recovery and activity recovery)

After achieving the resolution and recovery as per the guideline requirements, the following United State Pharmacopia (USP) validation parameters of the desired method shall be performed.

1 Linearity
2 Precision
3 Accuracy
4 Sensitivity
5 Assay reproducibility
6 Robustness

Once the method has been developed and validated, a validation report shall be prepared in terms of summary of methodology, type of Instrument, column type chemicals, solvents, reagents, salts, and all other tools used in the study.

All the statistical parameters of the results shall be tabulated, calculated and complied and the final report will be prepared as per the current API pharmaceutical Industry requirements.

UV-Vis Spectrophotometer

As per the nature of the solubility of the derivatives, suitable solvent will be selected. For different Embelin derivatives, the selected solvents will be used for the measure the maximum absorption wavelength. In this section the $\lambda_{max,}$ inter and intera molecular Hydrogen bonding and epsilon absorptions shall be determined.

Fourier Transform Infrared Spectroscopy (FT-IR)

The infra-red absorption spectrum of solid samples will be determined by Powder Diffusion method the liquid samples will be analysed by ATR method. The Finger print and the functional group regions shall be used to confirm the functional group of the derivatives.

All the respective FT-IR spectrum transmitted peaks shall be labeled and included in the final report.

Differential Scanning Calorimeter (DSC)

The DSC study will carried out for all the solid and liquid samples of the Embelin derivatives. All the thermal properties of the derivatives shall be studied in terms of melting, crystallisations, thermal reversible property and heat transitions properties.

All the respective DSC thermo grams shall be labeled for its thermal transitions and included in the final report.

Materials and Instruments: All the derivatives were received from Dr. Vijay Dattatray Gangan, for the proposed study. The derivatives were analysed by the Quality Control (QC) Analytical instruments (UV-Vis, FT-IR, HPLC and DSC) which being well maintained in terms of calibrations and periodic maintenance.

Methods: The analytical methods will be developed from the base of the molecule property and the related publication of the related molecules.

In this present work, Embelin has been isolated form Embelia ribes by using differential polarity of solvents using petroleum ether, chloroform, ethyl acetate, methanol and water. The crude chloroform extract was successfully chromatographed over silica gel followed re-crystallization using petroleum ether to yield pure Embelin which was diversified to its respective ether and ester derivatives.

These derivatives were unambiguously characterized using reference MNR and Mass spectroscopy. All these derivatives were checked for their novelty using Scifinder search and were found to be unique (totally new). However certain critical parameters has to be studied by using spectroscopy and chromatography instruments such as HPLC, UV-Vis, FT-IR and DSC; which is of prime importance in today's growing Active Pharmaceutical Industry.

These derivatives shall be analysed on HPLC for purity, related substance (by RRT). All the peaks will be well separated in a single HPLC acquisition. This method will be validated as per the pharmacopial[4] requirements.

Embelin derivatives will be analysed by UV-Vis, for the determinations of λ_{max} in different solvents. The electronic spectra and the nature of inter and intera molecular hydrogen bonding in different solvents shell be characterized.

The derivatives will be analysed on FT-IR by Powder Diffusion methods. The functional and finger print region peaks shall be characterized in terms of the functional group of the respective molecule.

The analysis by DSC will be described the with respect to various thermal prosperities in terms of glass transition, melting, diffusion, re-crystallization and degradation of the solid Embelin derivatives.

References

Bhavil et al (2005) A Validated Stability-indicating Reverse Phase HPLC Assay Method for the Determination of Memantine Hydrochloride Drug Substance with Uv-Detection Using Precolumn Derivatization Technique, *Analytical Chemistry Insights*, 5 37–45

Buchwald et al, (1980),Long-Term, Continuous Intravenous Heparin Administration by an Implantable Infusion Pump in Ambulatory Patients with Recurrent Venous Thrombosis, *Surgery* 88(4):507-516.

Chappell. et a1. (1994), Cellular Catabolism of Normal Very Low Density Lipoproteins via the Low Density Lipoprotein Receptor-Related Protein/Alpha 2-Macroglobulin Receptor is Induced by the C-Terminal Domain of Lipoprotein Lipase, *The Journal of Biological Chemistry* 269(27): 18001-18006.

Dai et al (2011), Natural IAP inhibitor Embelin enhances therapeutic efficacy of ionizing radiation in prostate cancer, *Am J Cancer Res* 1(2), 128-143.

Drent. et al. (1993), Lipase Inhibition: A Novel Concept in the Treatment of Obesity, *International Journal of Obesity and Related Metabolic Disorders* 17(4):241-244.

Faustinella et al (1992), Functional Topology of a Surface Loop Shielding the Catalytic Center in Lipoprotein Lipase, *Biochemistry* 31(32):7219-7223.

Ganesan et al (2010), Optimization of extraction conditions for embelin in Embelia ribes by UV - Vis. Scholars Research Library; *Archives of Applied Science Research*, 2 (2): 49-53

Giller et al (1992), Two Novel Human Pancreatic Lipase Related Proteins, hPLRPl and hPLRP2 Differences in Colipase Dependence and in Lipase Activity, *The Journal of Biological Chemistry* 267(23):16509-16516.

Goldberg et al (1982), Lipoprotein Metabolism during Acute Inhibition of Hepatic Triglyceride Lipase in the Cynomolgus Monkey, *The Journal of Clinical Investigation* 70(6), 1184-1192

Goldberg et al (1988), Lipoprotein Metabolism During Acute Inhibition of Lipoprotein Lipase in the Cynomolgus Mon key, *The Journal of Clinical Investigation* 81(2):561-568.

Gupta et al (1989), Effects of Embelin, an Antifertility Agent, on the Lipid Metabolism of Male Albino Rats, *Fitoterapia* 60(4):331-338.

Hixenbaugh et al (1989), Hepatic Lipase in the Rat Ovary, *The Journal of Biological Chemistry* 264(7):4222-4230.

Jaye et al (1999), A Novel Endothelial-Derived Lipase That Modulates HDL Metabolism, *National Genetics* 21(4):424 428

Jindal et al (2012), Antioxidant Activity of Ardisia crispa (Mata pelanduk), (Aktiviti Antioksida daripada Ardisia crispa (Mata pelanduk)), *Sains Malaysiana* 41(5), 539–545

Krapp et al. (May 1996), Hepatic Lipase Mediates the Uptake of Chylomicrons and Beta-VLDL into Cells via the LDL Receptor Related Protein (LRP), *Journal of Lipid Research* 37(5):926-936.

Langer et al (1984), Medical Applications of Controlled Release vol. I Classes of Systems. *CRC Press, Inc.: Boca Raton, FL., 4 pages. (Table of Contents).*

Langer et al (1983), Chemical and Physical Structure of Polymers as Carriers for Controlled Release of Bioactive Agents: A Review, *Journal of Macromolecular Science Reviews in Macromolecular Chemistry and Physics* C23(1):61-126

Langer, (1990), New Methods of Drug Delivery, *Science* 249(4976):1527-1533.

Liu et al (1994), Alteration of Lipid Profiles in Plasma of Transgenic Mice Expressing Human Lipoprotein Lipase, *The Journal of Biological Chemistry* 269(15):11417 11424

Lowe (1997). Molecular Mechanisms of Rat and Human Pancreatic Triglyceride Lipases, *The Journal Nutrition* 127(4):549-557

Maulik et al (2009), Screening of Embelia ribes for Antifungal Activity, International Journal of Pharmaceutical Sciences and Drug Research 1(3): 203-206

Nagarajan et al (2009), A validated RP-HPLC method for estimation of Oseltamivir in Pharmaceutical formulation, *Der Pharmacia Lettre*, 1 (1):162-168

Pandey et al (2011), Estimation of Embelin in Embelia tsjeriam-cottam Fruits by HPLC to Standardise Harvesting Time, *Indian Journal of Pharmaceutical Sciences*, March-April 2011, 216-219

Poojari et al (2010), Chemopreventive and Hepatoprotective Effects of Embelin on N-Nitrosodiethylamine and Carbon Tetrachloride Induced Preneoplasia and Toxicity in Rat Liver, *Asian Pacific Journal of Cancer Prevention*, Vol 11

Prakash et al (2011), In Vitro and In Vivo Anticancer Activity of Bacoside A from Whole Plant of Bacopa Monnieiri (Linn), *American Journal of Pharmacology and Toxicology* 6 (1), 11-19

Radhakrishnan et al (2011), A potential antibacterial agent Embelin, a natural benzoquinone extracted from Embelia ribes, *International Journal of Research and Reviews in Pharmacy and Applied science*, 2(1).257-267

Radhakrishnan et al (2011), Embelin-A natural potential cosmetic agent, *Journal of Applied Cosmetol*. (29), 99-107

Rakesh et al (2012), Development and validation of a RP-HPLC method for the simultaneous determination of Embelin, Rottlerin and Ellagic acid in Vidangadi churna, *Journal of Pharmaceutical Analysis*, 2(5),366-371

Ranganathan et al (1995), Tissue-Specific Expression of Human Lipoprotein Lipase, *The Journal of Biological Chemistry,* 270(l3):7l49-7l55

Rani et al (2010), Synthesis and Characterization of Ichlorobis (Emnbelinate) Cobalt (II), *International Journal of Research and Reviews in Pharmacy and Applied science*, 2(1).257-267

Rani et al (2010), Physico-chemical characterization of Dichlorobis (Embelinate) Zinc(II) complex, *Schlores Research Libreray Der Pharma Chemica*, 2(5), 89-95

Shimada et al (1993), Over expression of Human Lipoprotein Lipase in Transgenic Mice, *The Journal of Biological Chemistry* 268(24):l7924-l7929.

Singh et al (2012), Kinetics and Thermal Decomposition of Sm (III) complex with Embelin (2,5-Dihydroxy-3-Undecyl-p-Benzoquinone), *International Journal of Science And Nature*, 3 (3), 639-641

Srinivas, (2010), Anticancer and Antimicrobial Activity of Embelin Derivatives, *International Journal of preclinicaland pharmaceutical Research*, 1 (1), 35-38

Sudani et al (2011), Quantitative and Chromatographic fingerprint analysis of Embelia ribes churna formulations by HPLC method, *International Journal of Pharmaceutical & Biological Archives* 2(2), 657-663

Upadhyay et al (2008), Compounds for Treatment Of Lipase-Mediated Diseases, *US patent number US 7,355,055 B2*

Verger (1984), Pancreatic Lipases In Lipases. Bengt Borgstrom and Howard L. Brockman eds., Elsevier Science Publishers B.V.: Amsterdam, *The Netherlands*, pp. 83-150

Winkler et al (1979), Glycogen, Hyaluronate, and Some Other Polysaccharides Greatly Enhance the Formation of Exolipase by Serratia marcescens, *Journal of Bacteriology* l38(3):663-670

Winkler et al (1990), Structure of Human Pancreatic Lipase, *Nature* 343(6260):77l-774

Wong et al (1991), Domain Exchange: Characterization of a Chimeric Lipase of Hepatic Lipase and Lipoprotein Lipase, *Proceedings of the National Academy of Sciences of the United States of Americ,a* 88(24):ll290-ll294

Wong et al (2002), The Lipase Gene Family, *Journal of Lipid Research* 43(7):993-999

Yadav et al (1998), Purification and Characterization of a Region specific Lipase from Aspergillus terreus, *Biotechnology and Applied Biochemistry* 28(3):243-249.